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TITLE: Does the Loss of Stromal Caveolin-1 Remodel the Tumor Microenvironment by Activating Src-Mediated PEAK1 and PI3K Pathways?

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14. ABSTRACT

This study describes a new mechanism of intercellular communication originating from extracellular vesicles (EVs). We demonstrate that in the context of prostate cancer, EV populations isolated from human patients harbor AKT1 and that AKT1 kinase activity is sustained in these particles, nominating them as active signaling platforms. Consistently, active AKT1 in circulating EVs from the plasma of metastatic prostate cancer patients is detected predominantly in large, tumor-derived EVs, termed large oncosomes (LO). LO internalization induces reprogramming of human normal prostate fibroblasts, as reflected by high levels of α-SMA, IL6, and MMP9. In turn, LO reprogrammed normal prostate fibroblasts stimulate endothelial tube formation in vitro.

15. SUBJECT TERMS
PI3K, Prostate Cancer, Extracellular Vesicles

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1. INTRODUCTION

It has gradually become clear that the tumor stromal environment, also called the tumor microenvironment (TME), plays a crucial role in the initiation, progression and metastasis of cancer; hence, targeting the TME has emerged as a novel therapeutic strategy for cancer treatment (1). The TME consists of a compendium of cells (e.g., fibroblasts/myofibroblasts, vascular cells, immune cells, and etc.) along with the extracellular matrix (ECM) and extracellular molecules (2). Though many classic extracellular signaling molecules (e.g., hormones, peptide growth factors, and cytokines) are water-soluble, increasing evidence has shown that a variety of extracellular molecules are confined in extracellular vesicles (EVs) such as exosomes and microvesicles, and that EV-mediated intercellular communication plays an important role in cancer progression (3).

This project is testing the hypothesis that the **loss of Cav1 in prostate stroma promotes prostate cancer (PCa) progression and metastasis by activating the Src-mediated PEAK1 and PI3K pathways (Aim 1) and by regulating the release of certain extracellular proteins (Aim 2).** To this end we purified EVs derived from cancer cells with the PI3K pathway constitutively active in order to study its interaction with cells from the tumor microenvironment. We first address whether stromal cells, vascular endothelial cells, cancer associated fibroblasts and tumor cells could take up tumor-derived EVs. To infer whether EVs have the potential to reprogram cells from the tumor microenvironment we performed a transcription factor array analysis in stromal cells after EV uptake. During the second year of the funding period we will test whether stromal cells promote cancer metastasis *in vivo*.

Aim 1. Determine whether Cav1 loss activates the Src/PEAK1 and Src/PI3K pathways, whose cooperation enhances the dynamics of invadopodia-like protrusions and thus ECM degradation and EV secretion.

Aim 2. Determine whether Cav1 loss regulates the release of certain extracellular proteins, in water-soluble or EV-confined form, that are important for PCa progression

2.KEYWORDS

PI3K**,** Prostate Cancer**,** Extracellular Vesicles

3. ACCOMPLISHMENTS

What were the major goals of the project?

Training Goal 1: Training and educational development in prostate cancer research

Milestone: Presentation of project data at a national meeting
Target months: 24
Percentage of completion: 100%

Research Goal 1: Determine whether Cav1 loss activates the Src/PEAK1 and Src/PI3K pathways, whose cooperation enhances the dynamics of invadopodia-like protrusions and thus ECM degradation and EV secretion.

Milestones:

1) Determine whether Cav1 silencing activates the Src-mediated PEAK1 and PI3K pathways.

2) Determine whether targeting Src, PEAK1, or PI3K inhibits the dynamics of invadopodia-like protrusions, ECM degradation, and EV secretion.

3) Determine whether targeting Src, PEAK1, or PI3K in CAFs delay PCa growth *in vivo*.

Target months: 12

Percentage of completion: 100%

Research Goal 2: Determine whether Cav1 loss regulates the release of certain extracellular proteins, in water-soluble or EV-confined form, that are important for PCa progression

Milestones:

1) Determine whether EVs, EV-depleted conditioned medium, or both are the functional mediators of angiogenesis and tumor cell migration and invasion.

2) Identify the differentially secreted proteins using a quantitative proteomics approach

3) Determine whether manipulating the expression or activity of select proteins may regulate PCa progression *in vitro* and *in vivo*.

Target months: 24

Percentage of completion: 20%

What was accomplishing under these goals?

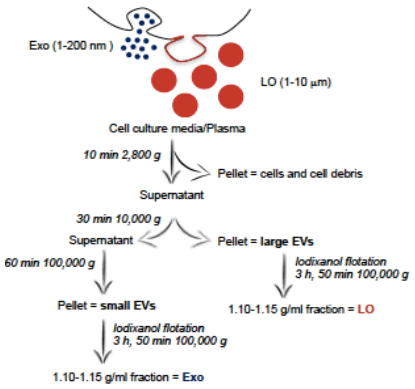
This study describes a new mechanism of intercellular communication originating from extracellular vesicles (EVs). We demonstrate that in the context of prostate cancer, EV populations isolated from human patients harbor AKT1 and that AKT1 kinase activity is sustained in these particles, nominating them as active signaling platforms. Consistently, active AKT1 in circulating EVs from the plasma of metastatic prostate cancer patients is detected predominantly in large, tumor-derived EVs, termed large oncosomes (LO). LO internalization induces reprogramming of human normal prostate fibroblasts, as reflected by high levels of α-SMA, IL6, and MMP9. In turn, LO reprogrammed normal prostate fibroblasts stimulate endothelial tube formation in vitro.

Major accomplishments during this funding cycle include:

1) Src and PI3K pathways activation promote EV secretion and AKT is selectively present in EVs

The loss of caveolin-1 is related with PI3K/Akt signaling pathway activation and increased shedding of EVs (4). Recent reports demonstrate that cells can shed

different populations of EVs, with a potential distinct role mediating intercellular communication. To test independently EVs ability to modify TME, we isolated small exosomes (Exo) and large oncosomes (LO) using optimized protocols for EV purification (figure 1).



We used a protocol based on differential centrifugation to separate LO from Exo, followed by flotation to exclude proteins and other EV-attached molecules (Fig. 1)

Figure 1. Schematic representation of the protocol used for purification of LO and exosomes (Exo) starting from conditioned media (CM).

A recent report identified AKT1 and other kinases in EVs circulating in blood from patients with different epithelial tumor types (5). Because AKT1 is frequently activated in patients with metastatic PCa as a result of genomic aberrations in the phosphoinositide 3-kinase (PI3K) pathway we analyzed the distribution of EV-bound AKT1 in both LO and Exo. We found that LO harbor p-AKT1^{Ser473} at significantly higher levels than Exo.

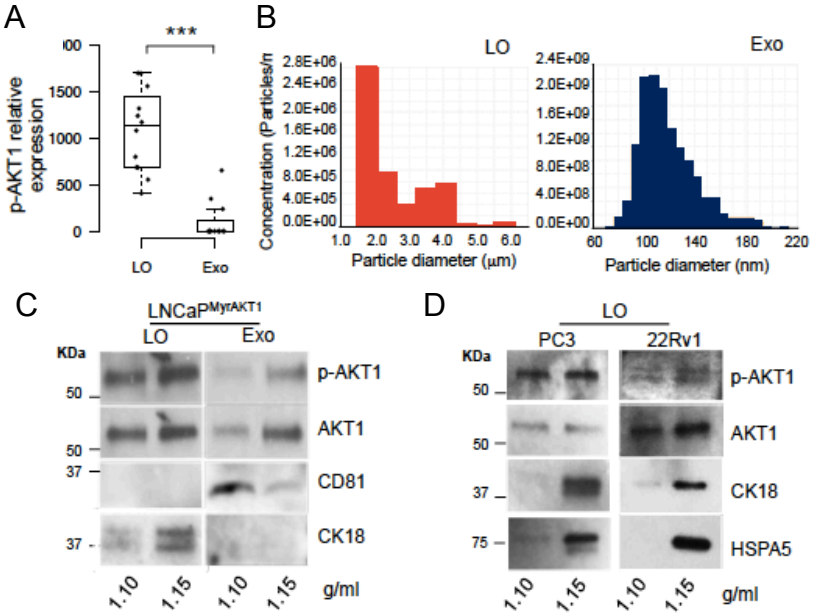


Figure 2. Large Oncosomes (LO) are EVs that harbor active AKT1. A, Protein lysates from LO and Exo purified from the plasma (500 μl) of patients with castration resistant prostate cancer (CRPC) (n=12) were blotted with a p-AKT1^{Ser473} antibody. p-AKT1^{Ser473} band intensity was normalized to protein content for each patient. Circulating LO carry significantly higher levels of active AKT1 than Exo (*** = $p < 0.002$). **C,** Tunable resistive pulse sensing (qNano) analysis of LO (left) and Exo (right) using NP2000 and NP100 membrane pores, respectively. **D,** LO and Exo

were purified from LNCaP^{MyrAKT1} cell media by gradient centrifugation (iodixanol), and protein lysates (10 μg) from the indicated fractions (1.10 and 1.15 g/ml density of the 10k and 100k pellets) were blotted with the indicated antibodies, including LO marker CK18 and Exo marker CD81. **D,** Equal amounts of protein from PC3 and 22Rv1 derived LO were blotted with the indicated antibodies.

2) Src pathway activation promotes plasma membrane blebbing and EV secretion

Next, we addressed whether the activation of Src through epidermal-growth-factor (EGF) and IL-6 stimulation (6) could increase the number of invadiopodia-like protrusions (Figure 3A and 3B). We tested the activation of Src pathways by western blot, phosphorylation of ERK is a downstream event (Fig 3B). Increased plasma membrane blebbing and shedding of EVs was detected after EGF incubation. Plasma membrane blebbing is associated with amoeboid motility and increased metastatic propensity (Figure 3). By time lapse video microscopy we detected the increase shedding of LO after IL-6 and EGF stimulation (Fig 3C).

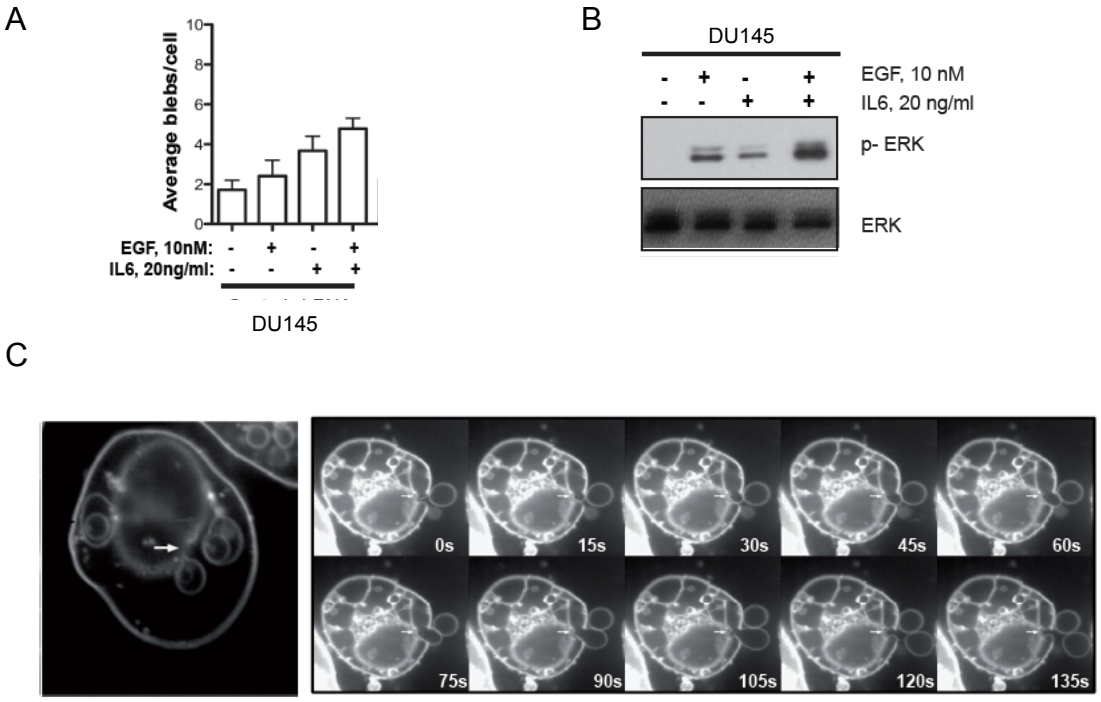
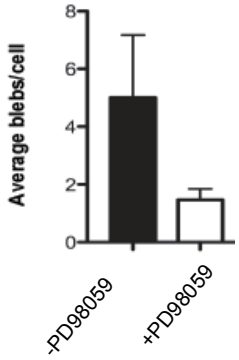


Figure 3. Increased plasma membrane blebbing and shedding of EVs through EGF and IL-6 stimulation. A, Average of blebs per cell in DU145 under EGF and IL-6 stimulation **B,** sustained ERK phosphorylation in response to EGF and IL-6, quantified by western blot in DU145 cells. **C,** Increased shedding of EVs under EGF stimulation in DU145 cells.

3) Inhibition of MAP kinase pathway prevents plasma protrusions

Next we used a selective inhibitor of MAP kinases, PD98059, to address whether we can prevent plasma membrane blebbing. The average number of blebs per cells was lower after incubation with PD98059 (Figure 3).



prevents plasma membrane blebbing. Average of blebs per cell in DU145 is prevented using PD98059 inhibitor.

4) LO are internalized by heterologous cells. EV uptake typically represents an important step for intercellular communication (7). In order to further investigate the consequences of LO uptake by cells from the microenvironment, we exposed immortalized WPMY-1 myofibroblasts to LO labeled with the fluorescent dye PKH26. LO uptake by target cells was quantitatively analyzed by flow cytometry (FACS) (Fig. 4A). Confocal imaging of FACS sorted LO-positive cells showed intact PKH26-labeled LO in the peripheral and perinuclear area (Fig. 4B). Increased PKH26 signal correlated with an increasing number of vesicles (Fig. 4C). Additionally, we found co-localization of PKH26 with MyrAKT1 (Fig. 4D), as detected with an HA-FITC antibody that binds with high specificity to the HA tag of the MyrAKT1 construct (8), which is expressed in the donor cells but absent in the target cells. These results suggest that the particles were intact LO rather than empty circular membrane structures capturing the lipid dye. We then determined whether cells other than myofibroblasts could also internalize LO. We tested normal human prostatic fibroblasts (NAF), human umbilical vein endothelial cells (HUVEC), CD8+ lymphocytes, and DU145 and LNCaP cancer cell lines. NAF are primary cells generated from prostatectomy tissues not associated with PCa. LO uptake varied among these cells, and was almost completely impaired in CD8+ lymphocytes (Fig. 4E), implying a selective mechanism of uptake. These observations suggest that LO enter target cells by a mechanism that might involve defined interactions between LO and the recipient cells.

To further rule out the possibility that LO uptake occurs by a passive fusion of EV and cell membranes, we incubated target cells with LO at 4^0C. This strategy has been previously used to inhibit ATP-dependent processes that are involved in EV endocytosis but not fusion (9). This approach efficiently prevented LO uptake (Fig. 5A) suggesting an active endocytic process. Due to their large size, we considered both phagocytosis and macropinocytosis as possible mechanisms, and tested the effect of known inhibitors of the major steps of these two processes on LO uptake.

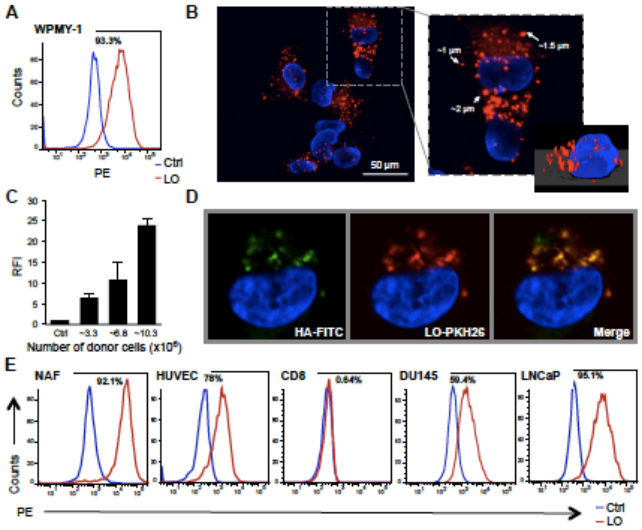


Figure 4: Internalization of LO by cells from the microenvironment. A, WPMY-1 fibroblasts were exposed to PKH26-labeled LO from LNCaP^{MyrAKT1} cells, or vehicle for 1h. The shift of the red line to the right, which is quantifiable, indicates LO internalization by the target cells. **B,** Cells positive for PKH26 were FACS-sorted and imaged by confocal microscopy demonstrating the presence of abundant vesicular structures in the LO size range. **C,** WPMY-1 cells were incubated with increasing doses of PKH26-labeled LO and then analyzed by FACS. Uptake rates, expressed as relative fluorescent intensity (RFI), correlate with LO doses. **D,** PKH26 positive WPMY-1 cells were sorted and stained with a HA-FITC antibody against the HA-tag on the MyrAKT1 construct. The two signals co-localize in internalized EVs. **E,** FACS analysis demonstrates variable uptake rate in the indicated cell lines exposed to PKH26-labeled LO.

The PI3K inhibitor, Wortmannin (WTN), and the actin polymerization inhibitor, cytochalasin-D (CYT-D) (10,11), typically used to block both phagocytosis and macropinocytosis (19), significantly perturbed LO uptake (Figure 5).

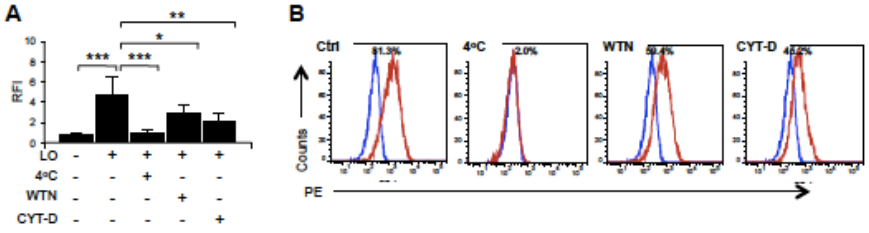


Figure 5. **A**, WPMY-1 fibroblasts were incubated with PKH26-labeled LO in presence or absence of Wortmannin (WTN) (1mM) or cytochalasin-D (CYT-D) (5mM), and then analyzed by FACS to quantify uptake inhibition. Bar plots show the mean of three biological replicates (*=p<0.05, **=p<0.02, ***=p<0.002). **B,** Representative FACS histograms of panel a.

To determine the relative contribution of these two processes, we used Dynasore-OH (Dyn) (12) and 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) respectively. The primary and most ubiquitous target of Dyn is dynamin 2 (DNM2), which plays a role in the first stages of phagocytosis, including actin

polymerization and augmenting of the membrane surface for particle engulfment (13,14). EIPA, which inhibits the Na+/H+ antiporter (15), is typically used to block macropinocytosis. LO uptake was significantly inhibited by Dyn but not by EIPA (Fig. 5A and 5B), suggesting that it occurs through a phagocytosis-like mechanism. The involvement of DNM2 in LO phagocytosis was further confirmed by a significant reduction in LO uptake upon transient silencing of DNM2 (Fig. 5 C, D and E).

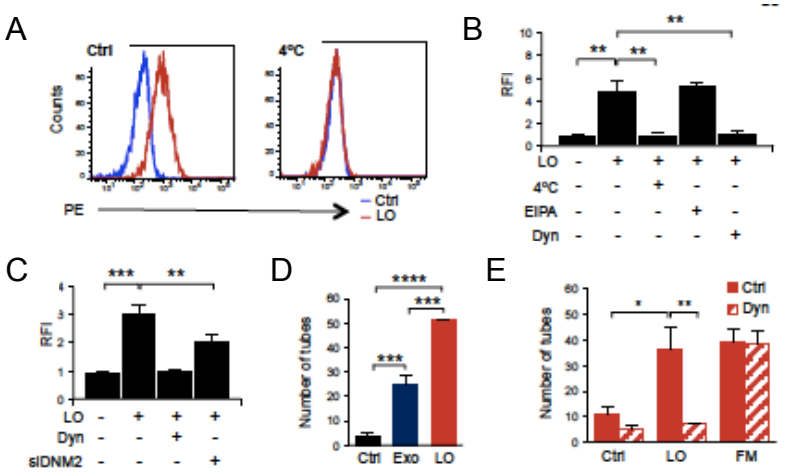


Figure 5. A, Treatment of WPMY-1 cells with LO at 4°C inhibits the uptake. **B,** LO uptake by WPMY-1 cells was significantly inhibited by Dynasore (Dyn) (20 μM) but not by 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) (50 μM). As expected, uptake was inhibited at 4°C. **C,** Transient silencing of DNM2 (siDNM2) in WPMY-1 cells resulted in a significant reduction of LO uptake. **D,** Human umbilical vein endothelial cells (HUVEC) were seeded on matrigel-coated wells and exposed to Exo or LO (20 μg/ml). The number of branched tubes was significantly altered by both LO and Exo. **E,** Dyn treatment prevented the LO-induced tube formation. Bar plots show the average of three biological replicates (* = $p < 0.05$, ** = $p < 0.02$, *** = $p < 0.002$, **** = $p < 0.000001$).

5) LO promotes tube branching ability in HUVEC

To determine whether the internalization is important for LO function, we employed tube formation assays, which have been previously used to show bioactivity of Exo (16) but have never been used to test LO function. Notably, LO stimulated a significant increase of the tube branching abilities of HUVEC. This effect was greater than that elicited by Exo, and was obtained with amounts of LO (5-20μg/ml) that are lower than those typically used for functional EV experiments (20-200 μg/ml) (17,18) (Fig. 6 C and 6D). Dyn treatment of HUVEC cells prevented LO-induced tube branching, but did not prevent the branching induced by full media (FM), which contains abundant soluble molecules that stimulate angiogenesis (Fig 6A and 6B). Collectively, these results indicate that LO enter the target cells through a phagocytosis-like mechanism, and that this is necessary for LO-mediated biological functions.

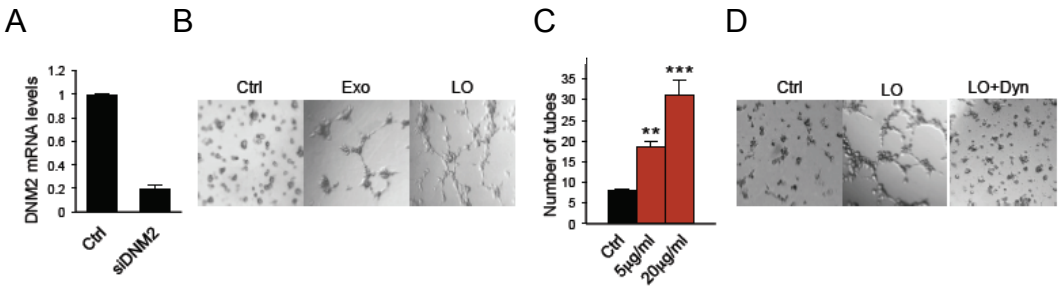


Figure 6. EVs induce angiogenesis. A) qRT-PCR in WPMY-q fibroblasts transiently silenced for DNM2, **B**) Representative images of the tube branching assay with HUVEC exposed to either Exo and LO from LNCAP ^{MyrAkt1} **C**) Tube branching assay with different doses of LO showing a dose-dependent response to LO treatment, which starts at 5 mg/ml. This dose corresponds to the use of LO from 10 door PCa cells to treat 1 recipient fibroblast, a result that is indicative of hogh biological potency. Bar plot shows the mean of three biological replicates **=p<0.02, ***=p<0.002). **D**) Representative images of the tube branching assay with Dyn. Bar plots show the mean of three biological replicates (8=p<0.05, 0.02, ***=p<0.002).

6) LO enhanced the expression pro-vascularization factors on NAF

Because it is known that tumor-activated fibroblasts release factors that can influence tube formation (19), and having observed a potent induction of tube branching in response to LO used directly to condition endothelial cells, we tested whether this effect in endothelial cells could be elicited by the secretions of fibroblasts that had internalized LO. Conditioned media (CM) from NAF pre-treated with LO induced a more significant increase in tube branching than Exo (Fig 7A). To understand the molecular basis underlying the LO-induced result on NAF, we tested changes in expression of factors that are upregulated in fibroblasts activated by cancer cells (18,20). LO treatment resulted in enhanced expression of interleukin 6 (IL6), matrix metalloproteinase 9 (MMP9) (Fig. 3B) and α-smooth muscle actin (α-SMA) (Fig. 7B). Conversely, transforming growth factor β1 (TGF-β1), MMP1, thrombospondin 1 (TSP1), which also have been recognized as markers of an activated, myofibroblast-like phenotype (18,21-24) were not altered (data not shown) suggesting that LO induce a distinct reprogramming of the fibroblasts, which results in a pro-vascularization phenotype.

7) LO activate the transcription factor MYC in NAF. Transcription factor (TF) activation might be an important mechanism underlying the responses of target cells to EVs (25). However, how frequently this happens and whether this phenomenon is specific for a given subpopulation of EVs, or for a given TF, has not yet been investigated. We thus tested if LO treatment perturbed TF activity, with the underlying hypothesis that this could be the mechanism modulating the effects described above. Nuclear extracts of fibroblasts exposed to LO or vehicle were tested for functional binding of TFs to DNA. We employed an activity array for TFs with a known role in somatic cell reprogramming (including EGR1, Nanog, SOX2, ETS, KLF4, MEF2, MYC, Pax6, TCF/LEF). Independent trials revealed reproducible enhancement of MYC binding to DNA in response to LO

(Fig 7B). To further validate this result, we measured MYC activity by examining the stimulation of MYC-dependent transcription. We used a luciferase assay that tests the activity of the cyclin-dependent kinase 4 (CDK4) promoter, which is regulated by MYC. Significant activation of this promoter was observed upon treatment with LO, but not with the same amount of Exo (Fig. 8D, $p < 0.05$).

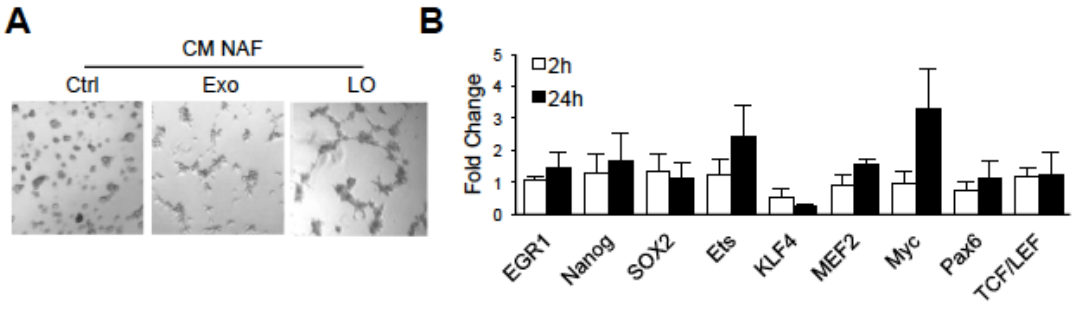


Figure 7) EVs promote angiogenic features. A) Representative images of tube branching assay in response to conditioned media (CM) from NAF previously incubated with Exo or LO. **B**) A transcription factor (TF) profiling array was applied to nuclear extracts of fibroblasts exposed to LO or vehicle for the indicated times to test FT activity in response to LO. Reporter activation was measured as relative luminescence activity in response to LO. Reporter activation was measured as relative to luminescence activity (RLU). The bar plot shows the ratio of the RLU between the treatment conditions and the control expressed as fold change.

This estimation was based on protein concentration (20 µg/ml), normalized to the number of cells. However, we reasoned that the array was composed of very few TFs, and a large-scale approach might be useful to unambiguously define the TF pathways involved in LO-mediated activation. RNA sequencing (RNA-seq) was carried out in NAF exposed to LO or vehicle to obtain an in-depth analysis of the transcriptome of these cells in response to LO. This analysis, performed in biological duplicate, identified 207 differentially expressed genes (DEG) (false discovery rate (FDR) < 0.1, fold change ≥ 1.5) in response to LO. Master regulator analysis (MRA) was then applied to the DEG set using TF-target interaction information collected from public databases. This allowed us to infer functional interactions between TFs and their target genes following a strategy we previously employed to identify important transcriptional regulators (26). 16 out of a total of 274 activated TFs emerged as strong putative TFs (empirical test p-value < 0.01 and hypergeometric test p-value < 0.01). The number of putative TFs that were activated by LO is relatively small (~6%), suggesting that modulation of gene expression is selective. MYC emerged as a highly activated TF in response to LO (Fig. 8E), confirming our initial results. Furthermore, NAF exposed to LO exhibited increased levels of fibroblast growth factor 2 (FGF2), glutaminase (GLS) and lactate dehydrogenase (LDH), which are known transcriptional targets of MYC (Fig. 8F). These results support an LO-dependent modulation of MYC activity in fibroblasts.

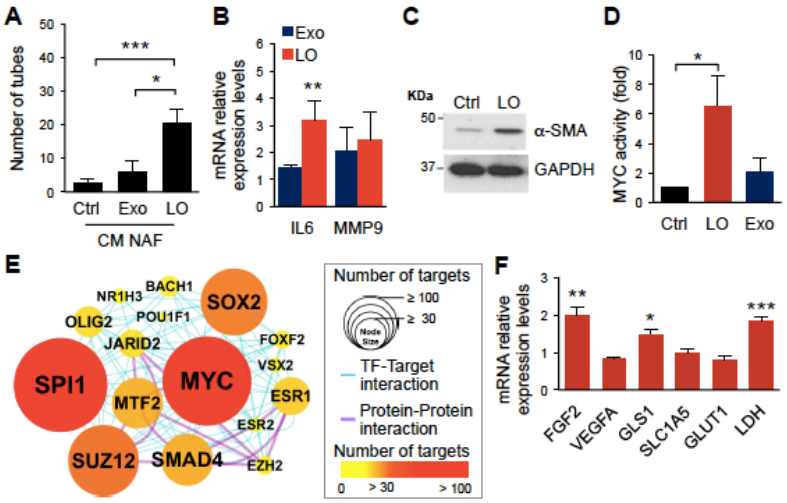


Figure 8) LO treatment of normal associated human prostatic fibroblasts (NAF) induces a MYC-dependent reprogramming. A, HUVEC cells were exposed to CM from NAF, previously incubated with LO and Exo. The CM from NAF pretreated with LO, but not Exo, induced tube formation. **B,** qRT-PCR of NAF exposed to LO or vehicle shows increased levels of IL6 and MMP9 mRNA in response to LO treatment. **C,** Immunoblot experiments demonstrated increased levels of α-SMA in NAF upon 24h exposure to LO. **D,** Luciferase activity of MYC regulated CDK4 promoter significantly increased in NAF exposed to LO but not Exo. **E,** Master regulator analysis (MRA) of differentially expressed genes (DEG) obtained after RNA-Sequencing in NAF treated with LO or vehicle. MYC is one of the most active TF in NAF in response to LO. TF network illustrating interactions between key TFs and the degree of influence to potential target genes among the DEGs (node size and color respectively). TFs with a large number of targets (> 105) is represented by big red nodes while TFs with smaller numbers of targets (< 50) are indicated with small yellow nodes. Cyan and purple connectors indicate TF-target and protein-protein interactions, respectively. **F,** qRT-PCR in NAF, exposed to LO or vehicle, shows increase levels of MYC targets in response to LO. **G,** Immunoblot analysis showing that MYC inhibition, using either the MYC inhibitor 10058F4 (MYC-i) (20 μM) or siRNA specific for MYC (siMYC) prevents LO-dependent induction of α-SMA. **H,** MYC inhibition (MYC-i, siMYC) induces a reduction of tube formation in response to LO. **I,** Luciferase activity of the MYC-regulated promoter in response to LO is inhibited by Dyn.

8) Key research accomplishments

- Activation of PI3K and Src pathways promotes shedding of LO
- Inhibition of MAP kinase pathway prevents plasma protrusions, involved in cancer cells motility
- LO are EVs that harbor active AKT1
- LO can be internalized by cells from the PCa microenvironment
- The internalization of LO can be prevented by inhibiting phagocytic-like events
- LO enhanced the expression of pro-vascularization factors on normal associated human prostatic fibroblasts
- LO treatment of normal associated human prostatic fibroblasts induces a MYC-dependent reprogramming

5) Conclusion

This is the first study testing the role of LO in educating the fibroblasts toward a tumor supportive function. We identified a novel AKT1/MYC signaling axis that originates from the tumor and reverberates to the stroma as a specific mediator of LO biological effects. Additional studies will further elucidate the function of LO in the modulation of the tumor microenvironment and identify additional nodes that could be targeted to prevent tumor progression and metastasis.

6) Other achievement

Stromal cells caveolin-1 silenced uptake higher percentage of tumor derived LO. Previous findings demonstrated that Cav-1 expression in the stroma can decline in advanced and metastatic prostate cancer, taking together this finding suggests that the loss of Cav-1 in stroma can facilitate the cells reprograming and tumor progression.

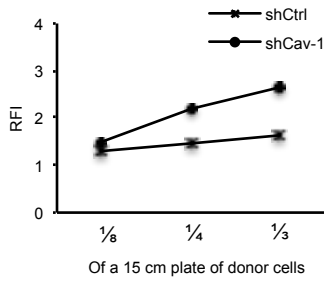


Figure 9. **LO are preferentially internalized by stromal cells with low Cav1**. Fibroblasts WPMY-1 Cav-1 silenced and controls using shRNA were exposed to increase concentration of LO tumor cells derived.

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What opportunities for training and professional development has the project provided?

I am a co-author of 2 published studies (see PRODUCTS section). I gave an oral presentation at the 2016 International Society of Extracellular Vesicles (ISEV). I gave presentations in lab meetings, journal club, and cancer biology workgroup meetings. I have substantive one-to-one discussions with the mentors several times per week and is in near-constant contact via e-mail, telephone or Skype. I have (and will continue) close communication with other senior investigators through many other routes, including (1) weekly joint lab meetings and (2) bi-weekly Cancer Biology Journal Club (organized by Dr. Kim). This is a very interactive community with open lines of communication across 8 nationally prominent prostate cancer research laboratories, where opinions, regents and data are continuously shared.

At this stage in the funding cycle, I going to submit a paper describing the EVs derived from metastatic cells and its possible clinical significance, which was DIRECTLY derived from this proposed study.

How were the results disseminated to communities of interest?

From this work, we demonstrated for the first time that LO circulates in blood from prostate cancer patients, containing active AKT1. This is a major discovery in the field, which has allowed collaborations with other nationally prominent prostate cancer research teams, including UCLA. This work elucidates of the function of LO in the modulation of the tumor microenvironment and identifies the nodes that could be targeted to prevent tumor progression and metastasis. Part of this work has been recently submitted to publication in Cancer Research journal.

What do you plan to do during the next reporting period to accomplish the goals?
A major objective of the second year of funding period is to test whether manipulating the expression or activity of select proteins may regulate PCa progression *in vivo*. We will also identify the differentially secreted proteins using a quantitative proteomics approach. We will apply a set of experimental and bioinformatics strategies to understand the function of LO in the tumor microenvironment and develop approaches directed toward targeting it.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
I have made an important conceptual and clinically relevant advance by characterizing the role of LO uptake in cells from PCa microenvironment. Consequently, this project is high impact and high reward, with potentially immediate opportunities to alter clinical practice if the classification scheme can be shown to have clinical utility.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report.

Changes that had a significant impact on expenditures
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations

Other publications, conference papers, and presentations.
Journal publications.

1. Minciacchi VR, Spinelli C, **Reis-Sobreiro M**, Cavallini L, You S, Zandian M, Li X, Chiarugi P, Adam RM, Posadas EM, Viglietto G, Freeman MR, Cocucci E, Bhowmick NA, Dolores Di Vizio. Prostate fibroblast reprograming induced by large oncosomes is mediated by MYC. Cancer Research (in press) 2016.
2. Ciardiello C, Cavallini L, Spinelli C, Yang J, **Reis-Sobreiro M**, de Candia P, Minciacchi VR, Di Vizio. Focus on Extracellular Vesicles: New Frontiers of Cell-to-Cell Communication in Cancer. Int J Mol Sci. 2016; 17(2):175.2.
3. Minciacchi VR, You S, Spinelli C, Morley S, Zandian M, Aspuria PJ, Cavallini L, Ciardiello C, **Reis-Sobreiro M**, Morello M, Kharmate G, Jang SC, Kim DK, Hosseini-Beheshti E, Tomlinson Guns E, Gleave M, Gho YS, Mathivanan S, Yang W, Freeman MR, Di Vizio D. Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles. Oncotarget. 2015; 6(13): 11327-41.

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
Poster presentation:

1. **Reis-Sobreiro M**, Morley S, Steadman K, Chen JF, You S, Yang W, Posadas E, Di Vizio, D, and Freeman M.R. Disruption of the LINC complex drives transition to the amoeboid phenotype. Cancer Research Poster Day Cedars Sinai Medical Center, Los Angeles, USA, 9 June 2016.
2. **Reis-Sobreiro M**, Morley S, Steadman K, Chen JK, You S, Posadas E, Di Vizio D, Freeman MR. Disruption of the LINC complex in cancer cells drives the genesis of extracellular vesicles with nuclear content. International Society of Extracellular Vesicles (ISEV), Rotterdam, The Netherlands, 2016 (Selected for oral presentation)
3. Minciacchi VR, You S, Yang W, Morello M, **Reis-Sobreiro M**, Spinelli C, Zandian M, Kim J, Rotinen M, Morley S, Freeman MR, Di Vizio D. Functional and quantitative proteomic analysis of two distinct populations of extracellular vesicles from the same cell source. International Society of Extracellular Vesicles (ISEV), Melbourne, Australia, 2014 (Oral presentation).
4. Minciacchi VR, Spinelli C, Morello M, You S, Yang W, **Reis-Sobreiro M**, Zandian M, Rotinen M, Morley S, Adam RM, Freeman MR, Di Vizio D. Large oncosomes are internalized and functionally modulate transcription factors in recipient cells. SELECTBIO meeting on Circulating Biomarkers, Boston, Massachusetts, USA, 2014 (Poster).
4. Minciacchi VR, Spinelli C, Morello M, You S, Yang W, **Reis-Sobreiro M**, Zandian M, Rotinen M, Morley S, Adam RM, Freeman MR, Di Vizio D. Large oncosomes are internalized and functionally modulate transcription factors in recipients cells. Annual

meeting of the American Association for Cancer Research (AACR), San Diego, USA, 2014 (Poster).
5. Minciancchi VR, Morello M, **Reis-Sobreiro M**, Spinelli C, Zandian M, Yang J, Rotinen M, Morley S, Adam RM, Freeman MR, Di Vizio D. Large oncosomes are internalized and modulate transcription factors in recipient cells. Annual meeting of the International Society of Extracellular Vesicles (ISEV), Rotterdam, The Netherlands, 2014 (Oral presentation).
6. Minciacchi VR, Spinelli C, Cavallini L, **Reis-Sobreiro M**, Zandian M, Adam RM, Posadas EM, Freeman MR, Cocucci E, Bhowmick NA, Di Vizio D. Large oncosomes reprogram prostate fibroblasts towards an angiogenic phenotype. Annual meeting of the International Society of Extracellular Vesicles (ISEV), Rotterdam, The Netherlands, 2016 (Oral).

Lecture:
Reis-Sobreiro M "Amoeboid motility and cancer metastasis" in the program Cell Biophysics in Physiology and Disease, held in University of California, Los Angeles, California (UCLA), March 2016.

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Mariana Reis-Sobreiro
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	
Contribution to Project:	Dr. Reis-Sobreiro has performed all the experiments
Funding Support:	The Urology Care Foundation Research Scholar Program

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS: Nothing to Report.

9. APPENDICES: Revised SOW

Statement of Work – 20/11/2014
Proposed Start Date 1 Sep, 2015

Site 1: Cedars-Sinai Medical Center [CSMC]
8700 Beverly Blvd.
Los Angeles, CA, 90048
PI: Mariana Reis-Sobreiro, PhD
Mentors: Michael R. Freeman, PhD
Dolores Di Vizio, PhD
Wei Yang, PhD

Training-Specific Tasks:

Major Task 1: Training and educational development in prostate cancer research *(only applicable to training award mechanisms)*	**Months**	**CSMC**
Subtask 1: Fine-tune bench skills for methodology optimization and experimental data generation	1-24	Dr. Reis-Sobreiro
Subtask 2: Attend weekly group meetings with other lab members and weekly one-on-one project meetings with three mentors	1-24	Dr. Reis-Sobreiro
Subtask 3: Present research at Cancer Biology Program group meetings	1-24	Dr. Reis-Sobreiro
Subtask 4: Attend lectures, mini-courses and workshops on cancer research and proteomics	1-24	Dr. Reis-Sobreiro
Subtask 5: Attend a national scientific meeting in relevant scientific field (e.g. AACR, IMPaCT)	13-24	Dr. Reis-Sobreiro
Milestone(s) Achieved: *Presentation of project data at a national meeting*	19-24	

Research-Specific Tasks:

Specific Aim 1: Determine whether Cav1 loss activates the		

Src/PEAK1 and Src/PI3K pathways, whose cooperation enhances the dynamics of invadopodia-like protrusions and thus ECM degradation and EV secretion.		
Major Task 1: Determine whether silencing Cav1 activates the Src-mediated PEAK1 and PI3K pathways.		
Subtask 1: Apply Western Blotting to determine the activity-related phosphorylation levels of Src, PEAK1, PI3K as well as several select substrates for these kinases. Cell lines used: WPMY-1 cells	1-3	Dr. Reis-Sobreiro
Subtask 2: Perform immunoprecipitation and in vitro kinase assay using a universal kinase assay kit (Abcam ab138879).	4-6	Dr. Reis-Sobreiro
Subtask 3: The differences in WPMY-1/shCav1 and WPMY-1/shCtrl cells will be compared.	7-8	Dr. Reis-Sobreiro
Milestone(s) Achieved: After silencing Cav1, the Src-mediated PEAK1 and PI3K pathways are activated.	8	
Major Task 2: Determine whether targeting Src, PEAK1, or PI3K inhibits the dynamics of invadopodia-like protrusions, ECM degradation, and EV secretion.		
Subtask 1: Use specific and potent inhibitors that are commercially available to inhibit the kinase activities of Src and PI3K Cell lines used: WPMY-1 and CAF cells	9-12	Dr. Reis-Sobreiro
Subtask 2: Apply RNA-mediated interference (RNAi) to reduce expression level of PEAK1.	9-12	Dr. Reis-Sobreiro
Subtask 3: Test whether targeting any of the three kinases in WPMY-1/shCav1 and CAF cells will inhibit the dynamics of invadopodia-like protrusions and ECM degradation.	12-16	Dr. Reis-Sobreiro
Subtask 4: measure EV secretion, I will use NanoSight nanoparticle tracking to quantify the number of secreted EVs	12-16	Dr. Reis-Sobreiro
Milestone(s) Achieved: After the inhibition of Src,PEAK1 or PI3K, the invadopodia-like protrusions, ECM degradation, and EV secretion are reduced.	16	
Major Task 3: Determine whether targeting Src, PEAK1, or PI3K in CAFs delay PCa growth *in vivo.*		
Subtask 1: Use RNAi to silence these kinases in WPMY-	17-20	Dr. Reis-

1/shCav1 cells, coculture kinase-silenced or control cells with PCa PC3 and ARCaP-M cells expressing fluorescence reporter gene, and implant them into 6-week old male nude mice (n=8) through tail vein injection.		Sobreiro
Subtask 2: Fluorescence-based noninvasive imaging will be used to monitor the metastasis of PC3 and ARCaP-M cells.	20-24	Dr. Reis-Sobreiro
Milestone(s) Achieved: After the inhibition of Src,PEAK1 or PI3K, the PCa will grow slower in vivo	24	
Specific Aim 2: Determine whether Cav1 loss regulates the release of certain extracellular proteins, in water-soluble or EV-confined form, that are important for PCa progression		
Major Task 1: Determine whether EVs, EV-depleted medium, or both are the functional mediators of angiogenesis and tumor cell migration and invasion.		
Subtask 1: Separate WPMY-1/shCav1- or WPMY-1/shCtrl-derived EVs from condition medium. I will perform angiogenesis, migration, and invasion assay.	1-6	Dr. Reis-Sobreiro
Milestone(s) Achieved: EVs, EV-depleted medium, or both promote(s) angiogenesis and tumor cell migration and invasion.	6	
Major Task 2: Identify the differentially secreted proteins using a quantitative proteomics approach.		
Subtask 1: Culture cells in the presence of AHA and stable isotope-labeled arginines. Isolate newly synthesized and secreted proteins.	7-10	Dr. Reis-Sobreiro
Subtask 2: Analyze purified proteins by LC-MS/MS Instrument used: mass spectrometer	11-12	Dr. Reis-Sobreiro
Subtask 3: Identify and quantify proteins.	13-14	Dr. Reis-Sobreiro
Milestone(s) Achieved: Get the mass spectrometry results	14	
Major Task 3: Determine whether manipulating the expression or activity of select proteins may regulate PCa progression in vitro and in vivo.		
Subtask 1: Prioritize the identified differentially secreted proteins. Select several candidates for functional assays.	15-16	Dr. Reis-Sobreiro

Subtask 2: For the *in vitro* studies, I will perform cell proliferation, migration, and invasion assays.	17-20	Dr. Reis-Sobreiro
Subtask 3: For the *in vivo* studies, I will perform tail vein injection as essentially described in the Major Task 3 of Aim 1.	21-24	Dr. Reis-Sobreiro
Milestone(s) Achieved: The expression or activity change of some candidate protein will promote or inhibit PCa progression	24	